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EFFECTS OF WR-2721 ON ENDOGENOUS SPLEEN COLONY FORMATION IN X-IRRADIATED MICE

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The effects of X-rays and WR-2721 ((S)-2-/3-aminopropylamino/ethylphosphorothioic acid) on hematopoietic injury and recovery were studied. Adult male Swiss mice were either whole-body X-irradiated or treated with WR-2721 before X-ray exposure. The in vivo endogenous spleen colony-forming unit assay was used. The grossly visible spleen colonies were counted and their areas were determined on the 12th day following X-irradiation only or pre-treatment of mice with WR-2721. Pre-irradiation administration of WR-2721 appeared to be effective in increasing hematopoietic stem cell survival. The effective protection of WR-2721 against X-ray induced radiotoxicity in the mouse hematopoietic system was shown.

Key words: WR-2721, X-rays, radioprotection, endogenous spleen colony formation

INTRODUCTION

The hematopoietic system is well known for its high sensitivity to ionizing radiation. Cytotoxicity of ionizing radiation on normal hematopoietic cells is an important problem (YOKOMICHI, 1972; MAC VITTIE, 1997; DEVI, 2003; SINGH et al., 2011). Therefore, a wide range of different compounds have been tested in experimental models to overcome the deleterious effects of ionizing radiation on hematopoietic cells (GUPTA et al., 2008; HAE et al., 2008; MANTENA et al., 2008a, 2008b). Walter Reed (WR)-2721 is one of the cytoprotective agents used against radiation-induced injury (HOSPERS et al., 1999; MAZUR, 2002; HOGLE, 2007; KOUVARIS et al. 2007).

Hematopoietic recovery depends on the percentage of residual hematopopietic stem cells. It is accepted that formation of endogenous spleen colonies is an indicator of hematopoietic stem cell proliferation. The hematopoietic recovery occurring after exposure of an organism to ionizing irradiation can be measured by the endogenous spleen colony-forming unit (E-CFUs) assay. The day 12 endogenous spleen colony formation in irradiated mice is used as a short-term quantitative in vivo assay for pluripotent hematopoietic stem cells (PATCHEN et al., 1990; SANDEEP and NAIR, 2011).

The present study was undertaken to determine the effects of WR-2721 and ionizing radiation

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on the mouse hematopoietic system. A possible influence of WR-2721 on the enhancement of hematopoietic reconstitution, following exposure of mice to X-rays, was analyzed.

MATERIALS AND METHODS

Animals

The adult male Swiss mice (Animal Breeding Unit, Cracow) employed in the experiments weighed from 27 to 30 g. All the mice were kept under constant environmental conditions with a 12/12 light/dark cycle. They were fed standard granulated chow and given drinking water *ad libitum*.

Exposure to X-rays

The mice were whole-body X-ray-irradiated, using a TUR-250 Roentgen apparatus (Institute of Nuclear Physics, Cracow). Irradiation conditions: 250 kV; 10 mA; exposure rate – 1.23 Gy/min, target distance – 448 mm; filters – 0.5 mm Cu, 0.5 mm Al. During exposure to a single dose of X-rays, the animals were placed in a well-ventilated acrylic box.

WR-2721 application

(S)-2-/3-aminopropylamino/ethylphosphorothioic acid (WR-2721, Amifostine) obtained from the Institute of General Chemistry, Warsaw Agricultural University, was dissolved in *aqua pro injectione*. The solutions of WR-2721 were freshly prepared, directly before treatment of mice. The intraperitoneal route of application was used in the experiments. The injected volume of the aminothiol solution was 200 µl per mouse.

Doses of X-rays and WR-2721

The mice were exposed to X-rays only, and treated with WR-2721 prior to X-irradiation, always at about 10.00 a.m. The mice were irradiated with sublethal and lethal single doses of X-rays from 5 Gy to 17 Gy. The aminothiol was given at a single dose of 400 mg/kg body weight, 30 min prior to exposure of mice to X-rays. This dose of WR-2721 represents two-thirds of the toxic LD_{50} lethal dose. The dose of the aminothiol given in the present investigations and the time interval between WR-2721 administration and irradiation coincide with those most often used in radioprotection (Santini and Giles, 1999; Mazur, 2000; Mazur et al., 2003).

Mortality of mice

The mortality of mice was analyzed during the 12-day period. The lethal dose ($LD_{50/12}$) for mice X-irradiated only and those pretreated with WR-2721 was determined and the dose reduction factor (DRF) was calculated.

Spleen colony assay

Twelve days following X-irradiation, and WR-2721 application before exposure to X-rays, the mice were euthanized by cervical dislocation. Their spleens were removed, fixed in Bouin's solution, and the grossly visible spleen colonies were counted. For morphometric studies, thin 4–5 µm sections of the spleens were prepared. These spleen sections were stained with hematoxylin and eosin. Using light microscopy and IBM Computer Scanning Systems, the areas of both the spleen and the formed endogenous spleen colonies were assessed. The ratio of the E-CFUs area to the spleen area was determined. The dose modifying factor (DMF) was calculated.

Statistical evaluation

Statistical significance of alterations in the number of E-CFUs between the particular groups of mice was evaluated by the analysis of variance and Duncan's new multiple range test.

RESULTS

The mortality of adult male Swiss mice exposed to X-irradiation only and those pre-treated with WR-2721 was determined (Table 2). The value of $LD_{50/12}$ for mice X-irradiated only and those treated with WR-2721 before irradiation was 7.4 Gy and 17 Gy, respectively. The value of DRF was 2.30.

TABLE 1. The effects of WR-2721 on the endogenous spleen colony formation in X-irradiated mice.

X-ray-dose	X-rays	WR-2721 + X-rays
(Gy)	Number of E-CFUs	Number of E-CFUs
5	confluent	confluent
6	confluent	confluent
7	8, 12, 13, 14, 15, 16 19.62 ± 2.77	confluent
8	7, 12, 13, 14, 15 2.17 ± 0.41	confluent
9	***	confluent
10	***	confluent
11	***	confluent
12	***	7, 8, 13, 14, 15, 16 15.80 ± 3.70
13	***	7, 8, 12, 14, 15, 16 12.75 ± 2.22
14	***	7, 8, 12, 13, 15, 16 9.33 ± 2.58
15	***	7, 8, 12, 13, 14, 16 6.29 ± 1.25
16	***	7, 12, 13, 14, 15 2.20 ± 0.45

*** not done

Statistically significant differences at $P<0.05$
Differences between groups of mice:
different from group of mice exposed to X-rays at a dose of 7 Gy – 7; 8 Gy – 8;
different from group of mice treated with WR-2721 prior to X-irradiation, at a dose of 12 Gy – 12; 13 Gy – 13; 14 Gy – 14; 15 Gy – 15; 16 Gy – 16.

TABLE 2. The mouse mortality and the ratio of E-CFUs area to the spleen area in mice exposed to X-rays only and those treated with WR-2721 prior to X-irradiation.

Experimental group	Mortality (%)	E-CFU area / spleen area (x 10^2)
7 Gy X-rays	21.43	39.37
8 Gy X-rays	62.50	5.87
WR-2721 + 12 Gy	0.00	26.51
WR-2721 + 13 Gy	0.00	17.91
WR-2721 + 14 Gy	16.00	13.94
WR-2721 + 15 Gy	25.00	10.47
WR-2721 + 16 Gy	37.00	5.78

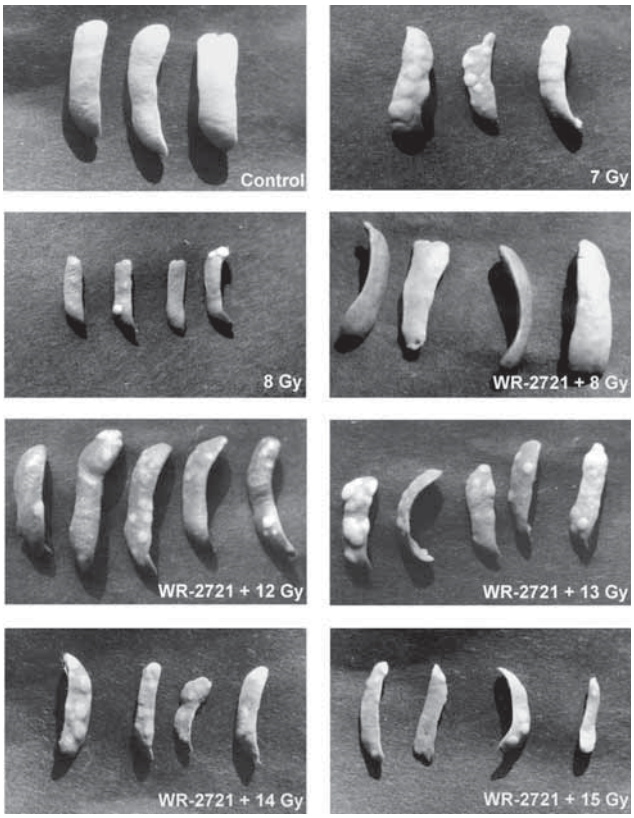

Fig. 1. The endogenous spleen colonies visible on the 12th day following X-irradiation only or pre-treatment of mice with WR-2721.

The effects of X-rays and WR-2721 applied prior to the exposure of mice to X-irradiation on the day 12 endogenous spleen colony formation were assessed (Table 1, Table 2, Fig.1). The day 12 E-CFUs formed in the spleens were shown (Fig.1).

The confluent spleen colonies were observed in mice exposed to 5 Gy and 6 Gy X-rays and in those treated with WR-2721 prior to X-irradiation at a single dose of 5 Gy, 6 Gy, 7 Gy, 8 Gy, 9 Gy, 10 Gy and 11 Gy. The greatest number of the grossly visible E-CFUs was found in mice exposed to X-rays only, at a dose of 7 Gy. Among mice pre-treated with WR-2721, the greatest number of the grossly visible endogenous spleen colonies was observed after the exposure of mice to 12 Gy X-rays, and declined with the increasing X-ray doses. The number of E-CFUs was greater in mice exposed to 7 Gy X-rays only than in those treated with both WR-2721 and 12 Gy X-rays. The number of the grossly visible E-CFUs found for mice X-irradiated only, at a dose of 8 Gy, was similar to that observed for the animals given WR-2721 prior to 16 Gy X-ray exposure. The patterns of changes in the ratio of the E-CFUs area to the spleen area appeared to be

similar to the patterns of alterations in the number of E-CFUs observed on the 12th day after X-irradiation alone and WR-2721 administration prior to X-ray treatment of mice. Based on the data of the present study, the obtained value of DMF was 2.

DISCUSSION

The results of the study have shown the effects of WR-2721 and X-rays on the mortality and the hematopoietic system of adult male Swiss mice, using $LD_{50/12}$ assay and the day 12 endogenous spleen colony forming unit assay. WR-2721, when given at a single dose of 400 mg/kg body weight, 30 min before X-irradiation, reduced the deleterious X-ray action on the mouse organism. The administration of WR-2721 before the lethal doses of X-irradiation enhanced survival of Swiss mice. Based on E-CFUs formation, it has been found that pre-irradiation application of WR-2721 enhanced hematopoietic reconstitution in X-irradiated mice. WR-2721 distinctly increased both the number of endogenous pluripotent hematopoietic stem cells and the area of the formed endogenous spleen colonies in mice exposed to the sublethal and lethal doses of X-rays. The radioprotective properties of WR-2721 against X-ray irradiation of Swiss mice have been demonstrated.

The mechanisms of action of WR-2721 on normal cells have not yet been completely elucidated. It is known that WR-2721 is a phosphorylated prodrug and its radioprotective action requires conversion to the active, free thiol compound WR-1065 (2-3/aminopropylamino/ethanothiol). WR-1065 is subsequently oxidized to the symmetric disulfide WR-33278. Various mechanisms or a combination of mechanisms of the radioprotective action of WR-2721 have been proposed: at the molecular level, by free radical scavenging, hydrogen donation, binding to critical biological targets, and mixed disulphide formation with endogenous thiols and thiol-containing proteins; at the biochemical-physiological level, by hypoxia, biochemical shock, and hypothermia, and at the organ level, by stimulation of cell population recovery (Griggs, 1998; Castiglione et al., 1999; Santini and Giles, 1999; Mazur, 2002).

The results of the present investigations have shown the effective radioprotection by WR-2721 against X-ray-induced injury to the mouse hematopoietic system. A better understanding of the radioprotective action of WR-2721 on hematopoietic cells is of key importance in cell biology, hematology, and radiotherapy.

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